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Gene therapy for murine glycerol kinase deficiency: Importance of murine ortholog

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Abstract

A glycerol kinase (Gyk) knock-out (KO) mouse model permits improved understanding of glycerol kinase (GK) deficiency (GKD) pathogenesis, however, early death of affected mice limits its utility. The purpose of this work was to delay death of affected males to investigate thoroughly their phenotypes. An adenoviral vector carrying the human (Adeno-XGK) or mouse (Adeno-XGyk) GK gene was injected into KO mice within 24 h of birth. Adeno-XGK did not change KO mouse survival time despite liver GK activity greater than 100% of wild type. However, Adeno-XGyk improved KO mouse survival time greater than two-fold. These investigations demonstrate that gene replacement therapy for Gyk KO mice is more efficacious using murine Gyk than human GK. These studies expand our understanding of GKD pathogenesis in the murine model, and show that while murine GKD is more severe than in humans, GKD mice have similar metabolic disturbances to affected humans with hypoglycemia and acidemia.
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Glycerol kinase (GK in human, Gyk in mouse) is an X chromosome-encoded enzyme that catalyzes the phosphorylation of glycerol to glycerol-3-phosphate [1]. This reaction has been recognized not only in mammals but also in various bacteria, fungi, and protozoa. Their amino acid sequences are highly conserved throughout evolution with ~50% sequence identity between mammals and bacteria [1–3]. GK in mammals is expressed at highest levels in liver and kidney, but is also expressed at lower levels in brain, intestinal mucosa, adipose tissue, and skeletal and cardiac muscles [1–4].

GK deficiency (GKD) in humans occurs as part of an Xp21 contiguous gene syndrome, complex GKD (cGKD), or as a consequence of mutations within the *GK* gene, isolated GKD (iGKD) [1,5–10]. cGKD has variable association with Duchenne muscular dystrophy and/or adrenal hypoplasia congenita. iGKD includes phenotypes ranging from asymptomatic hyperglycerolemia to symptomatic hyperglycerolemia with vomiting, acidosis, and central nervous system crises [1]. We have shown previously that among GKD patients with point mutations the GK genotype does not predict phenotype [10]. In that study, DNAs from six patients with symptomatic or asymptomatic iGKD were sequenced to identify point mutations, and GK activities were measured in transformed lymphocytes or fibroblasts. The GK activities ranged from a low of 5.53% to a high of

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12.7% of normal, and no correlations were found between levels of GK activity and patients' presence or absence of symptoms.

Gyk knock-out (KO) mice were established by targeted deletion [11]. Liver, kidney, and brown fat GK activities of these KO males were less than 5% of wild type mice. The Gyk KO males had growth retardation by day of life (dol) 2 and died by dol 3 or 4. The biochemical characteristics of mutant males included a >80-fold elevation in plasma glycerol and an approximately 3-fold increase in plasma fatty acid concentrations. The cause of death in the KO mice remained unclear [11], and, therefore, we determined that additional characterization of these Gyk KO males would be helpful to understand better the pathogenesis of the Gyk KO mice and the fidelity of that model with human GKD.

Gene therapy offers the possibility of replacing the deficient gene in the target organ. This approach has been used as therapy for a number of diseases. This therapeutic method may also be helpful in understanding mouse disease models that result in neonatal crisis or neonatal death. Although lipoprotein lipase and argininosuccinate synthetase deficient mice have shorter life spans than Gyk KO mice, gene therapy using recombinant adenovirus was effective in prolonging their survival [12,13].

Our goal was to obtain a better understanding of GK pathogenesis using Gyk KO mice; however, neonatal death limited our investigations. Therefore, the purpose of this study was to provide additional characterization of the Gyk KO mice, and to attempt to moderate their phenotype with gene replacement therapy using adenoviral *GK* and *Gyk* constructs. We observed that the murine Gyk KO model, though more severe than in humans, showed phenotypic fidelity at the metabolic level, with hypoglycemia and acidemia. We also found that the *Gyk* construct was far more efficacious than the *GK* construct, as measured by hepatic GK activities and survival times.

Materials and methods

Experimental animals and genotyping. Gyk KO mice, made using 129/SvJ embryonic stem cells and bred on a C57BL/6J mouse, were obtained from W.J. Craigen at Baylor College of Medicine [11]. They were bred and housed under an Animal Research Committee (ARC) approved protocol at the University of California, Los Angeles (UCLA). All experiments were performed according to a UCLA ARC approved protocol. Newborn mice from carrier females crossed with wild type (WT) males were injected within 24 h of birth with 40 μl of adenovirus via the superficial temporal vein using a 30 gauge needle. At ~45–48 h, or ~118–120 h after injection (dol 3 or 6, respectively), blood was collected and the liver, kidney, brown fat, and heart were harvested.

Identical amounts of Adeno-XGK or Adeno-XGyk virus were injected into KO and WT mice simultaneously. Tail or carcass genomic DNA was collected from mice to determine their genotype. Since in some cases, the carcasses were not recovered post-injection, Tables 2 and 3 show only those mice that had their genotypes confirmed.

Genotypes of Gyk KO mice were determined by PCR. Tail genomic DNA was extracted from injected or sacrificed mice using 2× lysis buffer (Applied Biosystems, Foster City, CA) and proteinase K (Sigma–Aldrich, St. Louis, MO). Primers for genotyping were neo-F2 (5′-gcgcatcgccttctatcgcc-3′) and GykR (5′-gttcaagactccacaccaacc-3′), which amplified the neomycin–Gyk junction fragment and primers for the normal allele, were GykF (5′-gatgccatgaatcgcgactgt-3′) and GykR. The PCR conditions were: 1× KlenTaq buffer, 5μM of neoF and mGykR primers, 2.5 μM of mGykF primer, 0.2 mM of each dNTP, 2.5 U of KlenTaq1 (Ab Peptides, St. Louis, MO), and ~0.1 μg of genomic DNA. The PCR cycling was as follows: 95 °C for 5 min to denature DNA; then 30 cycles of 94 °C for 30 s and 70 °C for 1 min; and a final extension of 72 °C for 5 min.

Assessment of blood gas, blood biochemical data, and urine glycerol level. Blood was collected from the external jugular vein with a pipette tip after the vein was opened with a needle. The collected blood for measuring blood gas and glucose levels was transferred into an Eppendorf tube containing 3 μl of lithium heparin (50 mg/ml) (Sigma–Aldrich). The i-STAT Portable Clinical Analyzer was used with the $^{CG}8^{+}$ cartridge (i-STAT, East Windsor, NJ) to measure pH, HCO_3^-, base excess and glucose from whole blood. The blood for plasma glycerol and free fatty acid analysis was collected and transferred into a 1.5 ml tube containing 3 μl of 0.5 mM EDTA–Na, then centrifuged to separate the plasma. The plasma samples were stored at −80 °C until plasma from 3 to 5 mice could be pooled and assayed at the UCLA Lipid Core. Urine was collected by putting pressure on the lower abdomen of the mice. SIGMA DIAGNOSTIC triglyceride (GPO-Trinder) was used to measure urine glycerol level as per the manufacturer's protocol.

GK assay. Liver, brown fat, heart, and kidney were taken from the injected mice at ~45–48 or ~118–120 h post-injection (dol 3 or 6, respectively). The tissues were transferred into 1.5 ml tubes containing 0.25 M Tris (pH 8.0), homogenized using a pellet pestle (Sigma–Aldrich), and then sonicated for 1 min each, eight times. The whole tissue lysate was centrifuged at 25,000*g* and the supernatant was used to measure GK activity by a radiochemical assay previously described, using 1 μg protein and a 20-min incubation [9]. For each tissue GK activity level, protein from two to three WT mice was extracted and their average activity was used to calculate the relative activity.

Preparation of recombinant adenovirus. The *GK* gene containing the human hepatitis C virus 5′ untranslated region (-cagaccgtgcatc-) [3] was cloned into the pShuttle plasmid (Clontech, Palo Alto, CA) with *Kpn*I and *Not*I sites (pShuttle hGK). The *Gyk* gene containing the 7 base pair sequence upstream from the *Gyk* start site was cloned into the pShuttle plasmid with *Nhe*I and *Xba*I sites (pShuttle Gyk). The fragments between *I-Ceu*I and *PI-Sce*I from pShuttle hGK and pShuttle mGyk were ligated into pAdeno-X adenovirus DNA (Adeno-XGK and Adeno-XGyk) (Clontech), which is a type 5 adenovirus deleted for the E1 and E3 region, according to the manufacturer's protocol. Adenovirus containing the *E. coli* β-galactosidase gene (Adeno-XLacZ) was prepared according to the Adeno-X Expression System (Clontech) manufacturer's protocol. The recombinant adenovirus vectors linearized with *Pac*I were transfected into 293 cells using SuperFect transfection reagent (Qiagen, Valencia, CA). The recombinant viruses were propagated and purified by ultracentrifugation through a $CsCl_2$ gradient then dialyzed with buffer [10 mM Tris (pH 8.0), 2 mM $MgCl_2$, and 5% sucrose (W/V)], and stored at −80 °C until injection. Injected recombinant adenovirus was diluted with phosphate buffered saline (PBS).

Statistical analysis. Data (blood glucose, blood gas analysis, plasma glycerol, and plasma free fatty acid) are shown as means ± standard deviation as determined using the Microsoft Excel Program. The *p* values were determined by *t* test analysis, using the Microsoft Excel Program. For statistical analysis, all samples determined to be unde-

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